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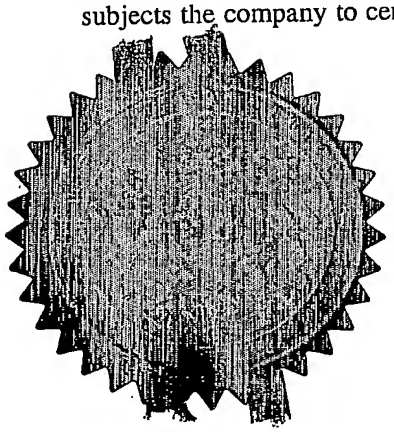

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1.	Your reference	VA/H-32622P1
2.	Patent application number *(The Patent Office will fill in this part)*	0220100.2
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	NOVARTIS AG LICHTSTRASSE 35 4056 BASEL SWITZERLAND
	Patent ADP number *(if you know it)*	7125487005
	If the applicant is a corporate body, give the country/state of its incorporation	SWITZERLAND
4.	Title of invention	Organic compounds
5.	Name of your agent *(If you have one)*	
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	B.A. YORKE & CO. CHARTERED PATENT AGENTS COOMB HOUSE, 7 ST. JOHN'S ROAD ISLEWORTH MIDDLESEX TW7 6NH
	Patents ADP number *(if you know it)*	1800001

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7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing (day/month/year)

8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *(see note (d))*	Yes

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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	ONE
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11. I/We request the grant of a patent on the basis of this application

Signature B. A. Yorke & Co. Date

B.A. Yorke & Co. 29 August 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

Mrs. E. Cheetham
020 8560 5847

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DUPLICATE

Organic Compounds

Field of the Invention

The present invention concerns use of a live strain of *Arthrobacter* in the preparation of a medicament to treat or prevent salmonid rickettsial septicaemia (SRS), and vaccine compositions based on these bacteria.

Background of the Invention

Piscirickettisia salmonis is a gram-negative obligate intracellular bacterium that causes systemic septicaemia (salmonid rickettsial syndrome, SRS) in salmonid fish. *Piscirickettsia*-like bacteria are now been recognized with increasing frequency in a variety of other fish species, from both fresh and salt waters around the world. Piscrickettsiosis and piscirickettsiosis-like diseases have affected aquaculture productivity, profitability, the species compatible with commercial rearing, and transportation of fish from site to site. The Chilean aquaculture industry alone attributes annual losses to salmonid piscirickettsiosis of $150 million. In Chile, the syndrome has led to a shift from the more commercially desirable coho salmon to the less desirable but more piscirickettsiosis resistant Atlantic salmon as the primary cultivated species.

Antimicrobials have been tested as a therapy for SRS, but without consistent success. Other suggested measures include attempts to reduce stress in the fish by reducing stocking density, and removing dead fish from tanks without delay. The most practical solution to the SRS epidemic would be to find an effective vaccine to prevent the disease in the first place. Inactivated bacterin preparations from *P. salmonis* have been shown to have some protective effect, and may be the only suitable option for co-administration in multivalent oil preparations, but are relatively expensive to produce on a commercial scale. Vaccines based on recombinant antigens from *P. salmonis* have not yet reached the marketplace.

Accordingly, there is an urgent need to make available a vaccine capable of significantly reducing mortalities due to piscrickettsiosis in fish. The present invention is based on the surprising discovery that an existing commercial vaccine product is remarkably effective in preventing the disease. This vaccine is marketed under the name "Renogen™" and

comprises a live, non-virulent strain of *Arthrobacter*. Currently, this vaccine is indicated to protect salmon and other farmed fish against bacterial kidney disease (BKD). The characteristics of this strain are disclosed in WO 98/33884, which is incorporated herein by reference.

Summary of the Invention

In one aspect of the invention there is provided use of live *Arthrobacter* cells in the preparation of a medicament for the treatment or prevention of piscirickettsiosis in fish. The preferred targets of the medicament are salmon exposed to risk of SRS infection.

In a second aspect of the invention there is provided a vaccine composition comprising live *Arthrobacter* cells and a killed bacterial immunostimulant, and a pharmaceutically acceptable carrier.

In a third aspect of the invention there is provided a method of treatment or prevention of piscirickettsiosis in fish comprising administering to fish in need of such treatment a vaccine comprising live *Arthrobacter* cells.

Detailed Description of the Invention

The Renogen™ vaccine has been in use for some time to combat Bacterial Kidney Disease (BKD) in salmonid fish. This vaccine is unique in that it is the first live culture to have been licensed for use in aquaculture, and comprises a live culture of *Arthrobacter sp. nov.*, deposited under Accession No ATCC 55921 with the American Type Culture Collection on 20 December 1996. *Arthrobacter* is not pathogenic to fish; nor is it the causative agent of BKD (which is *Renibacterium salmoninarum*).

It was observed on one site in the field that use of Renogen™ in a salmon population at risk of contracting BKD led to a dramatic reduction in mortality rates compared to untreated fish. SRS was also common on the site, which led the present inventors to speculate that Renogen™ may have conferred hidden protection against SRS as well as BKD.

In order to test this concept, tank-held fish were immunized with Renogen™ and subsequently challenged with *P. salmonis*, as described in Example 2. In the negative control group, which had received saline injections, nearly all the fish succumbed to SRS. The test groups that had received the Renogen™ vaccine exhibited extremely low mortality rates after 471 dd (degree days), amounting to between 88 and 100 relative percent survival (RPS). Even after 1441 dd (equivalent to one year in sea water) the test groups had a RPS of between 69 and 85%, compared to only 48.6% in the inactivated *P. salmonis* "gold standard" group.

Further evidence of the potential for vaccination with Renogen™ is demonstrated by the cross-reactivity of *P. salmonis* antigen when probed with rabbit polyclonal anti-*Arthrobacter* antibodies (Example 1).

We have shown that Renogen™ is more effective than any other known vaccine in preventing SRS. Live *Arthrobacter* bacteria are known to be able to enter cells and replicate for a limited period of time. The present inventors believe that this permits the antigen processing of both carbohydrate and protein antigens with sufficient homology to T-cell epitopes of *P. salmonis* to provide a high level of protection to direct challenge with virulent *P. salmonis*.

Renogen™ is based on a particular deposited strain of *Arthrobacter* (ATCC 59921). Species of the *Arthrobacter* genus are numerous and abundant in diverse habitats, including marine environments. Many *Arthrobacter* strains are available from commercial depositary institutions. It is not unduly burdensome on the skilled person to screen a selection of known strains or newly-isolated strains for the SRS immunogenic properties identified herein. Suitable strains can be identified by the experimental procedures described in Example 1 and Example 2.

The prime targets of the SRS vaccine of the invention are salmonid fish, including salmon and trout species, particularly Coho salmon (*Oncorhyncus kisutch*), Chinook salmon (*Oncorhynchus tshawytscha*), masu salmon (*Oncorhyncus masou*), pink salmon (*Oncorhynchus gorbuscha*), rainbow trout (*Oncorhynchus mykiss*), and Atlantic salmon (*Salmo salar*). However, any other fish species susceptible to piscirickettsiosis or similar

disease may benefit, such as, *Tilapia* sp., Black seabass (*Dicentrarchus sp.*), White seabass (*Atractoscion nobilis)*, grouper fish, cichlids etc.

The typical routes of administration of the vaccine are by injection into the peritoneal cavity (for larger fish), orally in feed, or by immersion in sea water or in fresh water. It is recommended that fish be 10 grams or greater in body weight for administration of the vaccine of the invention by intraperitoneal injection. For immersion or oral administration, a body weight of at least 2 grams is preferred.

The effective dosage of vaccine may vary depending on the size and species of the subject, and according to the mode of administration. The optimal dosage can be determined through trial and error by a veterinarian or aquaculture specialist. A suitable dosage range may be from about 10^2 to 10^9 cfu per unit dose, preferably about 10^4 to 10^7 cfu per unit dose. Preferably a single dosage unit is administered to the fish to be treated. Smaller fish may benefit from a dose of about 10^4 to 10^7 cfu/ml with dip (immersion) administration, for instance with a contact time of about 60 seconds.

Typically, vaccines are prepared as liquid solutions or suspensions for injection or delivery in water. For instance, a liquid emulsion or emulsifiable concentrate can be prepared in order to be added to a water tank or bath where the fish are held. Solid (e.g. powder) forms suitable for dissolution in, or suspension in, liquid vehicles, or for mixing with solid food, prior to administration may also be prepared. Preferably the vaccine is a lyophilised culture in a ready to use form for reconstitution with a sterile diluent. For instance, lyophilized cells may be reconstituted in 0.9% saline (optionally provided as part of the packaged vaccine product). Liquid or reconstituted forms of the vaccine may be diluted further in a small volume of water (e.g. 1 to 10 volumes) before addition to a pen, tank or bath. The pharmaceutical vaccine compositions of the invention may be administered in a form for immediate release or extended release.

Pharmaceutically acceptable carriers or vehicles include conventional excipients, and may be, for example, solvents such as water, oil or saline, dextrose, glycerol, wetting or emulsifying agents, bulking agents, coatings, binders, fillers, disintegrants, diluents, lubricants, pH buffering agents, or conventional adjuvants such as muramyl dipeptides,

avridine, aluminium hydroxide, oils, saponins, block co-polymers and other substances known in the art.

In a preferred embodiment the *Arthrobacter* vaccine of the invention comprises an immunostimulant, preferably a killed bacterial preparation. Suitable examples include: "Peptimune" (a heat-killed *Arthrobacter* culture) and "Ultracorn" (ultrasonicated *Corynebacterium cutis* lysate). An optimal dosage of killed bacterial immunostimulant is (per unit dose) 1 to 100 µg, preferably in the range 5 to 50 µg, and ideally at least 12 µg of cellular matter.

In some instances it may be desirable to combine the Renogen™ vaccine of the invention with a conventional SRS vaccine (*P.salmonis* bacterin or recombinant antigen vaccine or nucleic acid vaccine) in a combination vaccine, or in a kit comprising both components for separate, sequential or simultaneous administration, for treatment or prevention of SRS.

Examples

Example 1 Cross-reactivity of *P.salmonis* antigen with anti-*Arthrobacter* polyclonal antibodies

Approximately 25µg of triple-washed *P. salmonis* bacterial cells harvested from CHSE-214 cell culture were loaded on a gel and the proteins were separated out by gel electrophoresis. A Western blot of this gel was made in the conventional manner. The blot was incubated with 20µl of rabbit anti-*Arthrobacter* polyclonal antibodies for 45 minutes in 15ml of 1% casein tris-borate saline (cTBS). The blot was then exposed to 5µl of goat anti-rabbit immunoglobulin alkaline phosphatase (GAR-AP), and developed. Several proteins were highlighted on the blot, indicating that anti-*Arthrobacter* protein antibodies have a strong affinity to certain *P. salmonis* proteins. This result was also confirmed on a 2D Western blot.

This experiment shows that certain *P.salmonis* and *Arthrobacter* proteins cross-react, indicating that these *Arthrobacter* proteins can prime the immune system to produce antibodies potentially capable of recognizing and protecting against *P. salmonis* virulent bacteria.

Example 2

Coho salmon (n=110 per treatment group, mean weight 10 g) were maintained under normal husbandry conditions in tank water according to standard operating procedures at 12 °C. Following one week of acclimatization Groups 1, 2 and 3 were vaccinated intraperitoneally with 0.1ml of 10^5, 10^6, and 10^7 cfu/dose, respectively, of Lyophilized *Arthrobacter sp. nov* cells (Renogen) reconstituted in saline diluent. Groups 4 and 5 were treated in an identical manner to Group 1, but with the addition of 12.2 µg and 50 µg per dose, respectively, of the immunostimulant "Peptimune" in the saline diluent. Peptimune is a preparation of heat-killed *Arthrobacter* grown in liquid culture (MTSB broth) to a cell density of >1xE9, and standardized by protein assay to administer 12 and 50 µg per dose. Groups 6 and 7 were positive controls vaccinated with *P. salmonis* bacterin. The bacterin was prepared from the supernatant of a *P. salmonis* type strain LF-89 infected CHSE-14 cell culture using 0.125% formalin at 4°C over a minimum 72h period. U/F concentration was employed and the concentrated supernatant was used to incorporate 8 µg (protein) per 0.1ml dose. The bacterin vaccine was delivered with Ultracorn (Virbac, France) at 20 (Group 6) and 100 µg (Group 7) per fish. Ultracorn is an immune stimulant based on an ultrasonicated *Corynebacterium cutis* lysate. The antigens were emulsified with an equal volume of mineral oil adjuvant prior to injection. The negative control group (Group 8) received an injection of saline.

Table 1 summarizes the treatment groups (dose volume (0.1 mL per fish) for 20 mls):

Group	Treatment	Antigen Concentrate (ml)	Ultracorn (20 mg/ml)	Saline (ml)	Oil Adjuvant (ml)
1	10^5cfu Renogen™	nil	nil	1vial/1000 ml	
2	10^6cfu Renogen™	nil	nil	1vial/in one ml (99 ml saline)	

3	10^7cfu Renogen™	nil	nil	2 vials/2 ml (18 ml saline)	
4	10^5+100 ml (12.2 ug per dose Peptimune)			as 1	
5	10^5+ 400 ml (50 ug per dose Peptimune)			as 1	
6	Ps1-U 20 µg -3x	3	0.2	6.8	10
7	Ps1-U 100 µg -3x	3	1.0	5.8	10
8	Saline			0.1	

Challenge method

At 471 and 1441 dd (degree days) following vaccination, duplicate groups of 25 fish per treatment were challenged with virulent *P. salmonis* by intraperitoneal injection. Virulent *P. salmonis* was cultured on CHSE-14 cells for a minimum of 2-3 week old supernatants of culture reaching at least 50% CPE. The virulent *P. salmonis* injections were given at 10^{-2} dilutions or more at 0.1 ml per fish (n=25). Challenged fish were maintained at 12°C.

Before termination of the challenge 1, 10 fish from the surviving populations of Group 1, 7 and 8 (only 8 fish were survivors in this group) were sacrificed and a splenic and renal tissue sample of 0.5 g was taken, homogenized and diluted in 10 ml of tissue culture medium. A $TCID_{50}$ was determined on 96 well plates containing confluent CHSE-214 cells.

RESULTS AND DISCUSSION:

Table 1: Mortality during the 28 d safety test, maintained at 9-12 °C through-out the safety and pre-challenge period.

Group	Treatment	Tank	Loss per treatment (N)	Total (N)	% Mortality
1	Renogen™ 10^5 dose	I1	0	110	0
2	Renogen™ 10^6 dose	I2	0	110	0
3	Renogen™ 10^7 dose	I3	7	110	6.3
4	Renogen™ 10^5 dose +12.2 µg Peptimune	I4	1	110	0.9
5	Renogen™ 10^5 dose +50 µg Peptimune	I5	4	110	3.6
6	Inactivated SRS/U20/Oil	I6	0	110	0
7	Inactivated SRS/U100/Oil	I7	0	110	0
8	Saline	I8	0	110	0

During the safety study, it was observed that fish in Group 3 suffered some loss (6.3%) nearing the end of the 28 d safety period. The lab investigator treated all fish in the population with a three day formalin treatment for bacterial gill disease. Mortality (3.6%) in Group 5 was recorded during the initial three day period pv, indicating that the inclusion of Peptimune as 40% of the diluent was somewhat toxic. No positive plates were cultured from

the losses during the safety period, either for the live vaccine strain, or any incidental bacterial cultures.

Table 2: Cumulative Mortality and Relative Percent Survival of Coho salmon (mean weight 10 g) 471 dd post-vaccination with *Arthrobacter sp. nov* cells (Groups 1-5), Inactivated SRS vaccines, or saline when challenged with virulent *P. salmonis* by intraperitoneal injection (3 x $10^{2.9}$ per fish) at 12 °C.

Group	Treatment	Tank	Loss per duplicate tank (N)	Total	Loss per treatment	% Mort	RPS
1	Renogen™ 10^5 dose	L1, L2	0/25, 1/25	50	1/50	2	97.6
2	Renogen™ 10^6 dose	L3, L4	1/26, 0/24	50	1/50	2	97.6
3	Renogen™ 10^7 dose	L5, L6	2/25, 3/25	50	5/50	10	88.1
4	Renogen™ 10^5 dose +12.2 µg Peptimune	L7, L8	0/25, 0/25	50	0/50	0	100
5	Renogen™ 10^5 dose +50 µg Peptimune	L9, L10	0/25, 0/25	50	0/50	0	100
6	Inactivated SRS/U20/Oil	L11, L12	9/25, 12/25	50	21/50	42	50.0
7	Inactivated SRS/U100/Oil	L13, L14	7/25, 6/25	50	13/50	26	69.1
8	Saline	L15, L16	19/25, 23/25	50	42/50	84	----

At 471 dd post-vaccination, fish in Group 1 had a relative percent survival (RPS) of 97.6, a high level of protection from direct infection with *P. salmonis* over 32 days, where mortality in the saline control group was 84%. This compared favourably to the protection garnered from vaccination with the standard inactivated vaccines (Groups 6 and 7), that showed RPS values of 50 and 69% respectively.

$TCID_{50}$ Analysis of Surviving Fish in Group 1, 7 and 8.

Level of SRS infection in the tissue samples of the surviving fish from the 471 dd challenge (n=7-10), 32 days post-infection :

Group	Treatment	% of fish $TCID_{50}$ 10^2/mL	Mean $TCID_{50}$
1	Renogen™	20	$10^{4.5}$/mL
7	SRS/U/oil	44	$10^{4.6}$/mL
8	Saline	50	$10^{4.7}$/mL

The $TCID_{50}$ of the fish sampled from the Renogen™ group was lower than the inactivated vaccine group, and both were lower than the saline controls. This is not of apparent clinical relevance, as the contribution of the high titre groups negates the lower infective dosages when averaging. However, the Renogen™ group did have the lowest percent positives (<20%) as samples with less than 10^2 were considered not to be clinically infected with SRS. This compares to the same samples from the saline control group where 50% of the fish were positive for SRS, and favourably to the inactivated vaccine group with 44% of the fish positive for SRS.

Table 3: Cumulative Mortality and Relative Percent Survival of Coho salmon (mean weight 10 g) 1441 dd post-vaccination with *Arthrobacter sp. nov* cells (Groups 1-5), Inactivated SRS vaccines, or saline when challenged with virulent *P. salmonis* by intraperitoneal injection (TCID $3 \times 10^{2.9}$ per fish) at 12 °C.

Group	Treatment	Tank	Loss per duplicate tank (N)	Total	Loss per treatment	% Mort	RPS
1	Renogen™ 10^5 dose	L1, L2	8/25, 3/25	50	11/50	22	69.4
2	Renogen™ 10^6 dose	L3, L4	2/24, 3/25	49	5/49	10.2	85.8
3	Renogen™ 10^7 dose	L5, L6	3/19, 2/19	38	5/38	13.2	81.7
4	Renogen™ 10^5 dose +12.2 µg Peptimune	L7, L8	4/25, 5/25	52	9/52	17.2	76.1
5	Renogen™ 10^5 dose +50 µg Peptimune	L9, L10	2/24, 5/24	48	7/48	14.6	79.7
7	Inactivated SRS/U100/Oil	L11, L12	10/23, 7/23	46	17/43	37	48.6
8	Saline	L13, L14	20/25, 16/25	50	36/50	72	----

Note: back-up fish in Group 6 intended for the long term efficacy study were lost due to accidental shut-off of water flow in this tank (I7).

After an elapsed period of 1140 dd, the durational response of the protection observed at the earlier test period (471 dd) was assessed. Results of the second challenge where a level of 72% mortality was observed in the saline control group indicate that the level of protection is still high with Renogen™ treated fish (69.4% RPS), with some indication that a higher dosage may improve the long term protection (10^6 and 10^7 cfu/dose had RPS of 85.8 and 81.7 respectively). The addition of the immunostimulant Peptimune at 12 and 50 µg to the diluent provided a slight improvement to the efficacy of the product at dose (76.1 and 79.7 %

respectively). The accidental loss of the standard reference vaccine (group 6) allowed for comparison to Group 7 only, and this group had an RPS of 48.6%.

CONCLUSIONS:

Renogen provided significant protection against direct challenge with *P. salmonis* at 471 dd and at 1441 dd post-vaccination. The vaccine was superior to the protection provided by the standard oil vaccine. We were able to demonstrate that fewer surviving fish in the Renogen™ group were clinically infected with *P. salmonis*. The study demonstrates that *Arthrobacter sp. nov.* live vaccine provides a high degree of protection against *P. salmonis* infection, and that the protective effect is shown to be long-term.

Claims

1. Use of *Arthrobacter* cells in the preparation of a medicament for the treatment or prevention of piscirickettsiosis in fish.

2. Use according to claim 1 wherein the fish are salmonid fish.

3. Use according to claim 1 or claim 2 wherein the piscirickettsiosis is salmonid rickettsial septicaemia (SRS).

4. Use according to any preceding claim wherein the *Arthrobacter* cells are live.

5. Use according to any preceding claim wherein the *Arthrobacter* cells are from the strain deposited under accession number ATCC 55921, or any other *Arthrobacter* strain having similar immunogenic properties to this strain.

6. Use according to any preceding claim wherein the fish are Coho salmon (*Oncorhyncus kisutch*).

7. A vaccine composition comprising live *Arthrobacter* cells and an immunostimulant comprising killed bacterial cell material, with a pharmaceutically acceptable carrier.

8. A vaccine composition according to claim 7 wherein said cell material is from killed *Arthrobacter* cells.

9. A vaccine composition according to claim 7 or claim 8 wherein the *Arthrobacter* cells and optionally the killed bacterial cell material are from the strain deposited under accession number ATCC 55921, or any other *Arthrobacter* strain having similar immunogenic properties to this strain.

10. A vaccine composition comprising live *Arthrobacter* cells and further comprising at least one other immunogen.

11. A vaccine composition according to claim 10 wherein said immunogen is selected from the group consisting of: a bacterin prepared from *P. salmonis*; a recombinant *P. salmonis* antigen; and a nucleic acid vector carrying an expressible *P. salmonis* antigen.

12. A kit comprising a first vaccine comprising live *Arthrobacter* cells, and a second vaccine comprising an immunogen selected from the group consisting of: a bacterin prepared from *P. salmonis*; a recombinant *P. salmonis* antigen; and a nucleic acid vector carrying an expressible *P. salmonis* antigen; for separate, sequential, or simultaneous administration to fish.

Abstract

A vaccine based on live *Arthrobacter* cells is effective in preventing piscrickettsiosis in fish.